THE INACTIVATION OF FERTILIZIN AND ITS CONVERSION TO THE "UNIVALENT" FORM BY X-RAYS AND ULTRAVIOLET LIGHT

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Recently Tyler (1941) has shown that the substance known as fertilizin which is obtained from the eggs of certain marine invertebrates and which has the property of agglutinating species sperm, may exist in non-agglutinating form. Thus, fertilizin (egg water) of *Strongylocentrotus purpuratus,* when subjected to appropriate heating or enzymatic digestion, lost its ability to agglutinate, but retained its ability to combine with the sperm. Tyler has proposed the term, "univalent fertilizin," for such non-agglutinating substances. They are readily demonstrable since their addition to sperm inhibits subsequent agglutination by ordinary fertilizin of the same species.

Several workers have shown that the sperm agglutinating power of *Arbacia* egg water can be destroyed by X-rays. Richards and Woodward (1915) reported an initial rise and subsequent fall in sperm agglutinin titer with prolonged irradiation. More recently, Evans (1940), and Evans, Beams and Smith (1941) have shown that the egg jelly of *Arbacia* is dissolved by X-rays and that irradiated egg water will not give the Janus green reaction (Harvey, 1939) nor will it agglutinate species sperm.

It appeared possible that univalent fertilizin might be produced during treatment of fertilizin with X-rays. The present work was done primarily to examine this possibility. The results show that it does indeed occur. In the course of the work further quantitative data were obtained on the X-ray inactivation of fertilizin in terms of Roentgen units, fertilizin concentration and sperm concentration. In addition the possible action of another type of radiation, namely ultraviolet light, in inactivating fertilizin and producing univalent fertilizin was investigated. Here, also, the results were positive.

Material and Methods

The sea urchin, *Arbacia punctulata,* was used exclusively in the experiments with X-radiation, while *Strongylocentrotus purpuratus* was

used in the experiments with ultraviolet light. Eggs and sperm were obtained by opening the animals and allowing them to shed into stender dishes. Fertilizin was prepared by removing the supernatant sea water from a suspension of eggs which had stood overnight in the refrigerator, or by extracting eggs in acid sea water (Tyler and Fox, 1940) and readjusting the pH of the extract. All sperm suspensions were made from "dry sperm" (sperm collected from the gonopores in dry stender dishes). The concentrations of sperm are given as percentages of this dry sperm. The presence or absence of an agglutination reaction was determined by microscopic examination two to four seconds after mixing the solutions and sperm suspensions.

X-radiation in all cases was done in waxed paper cups at 5,600*r*/minute.

Ultraviolet radiation was given in open stender dishes. Controls were run in open dishes placed at the same distance (4.5 cm.) from the radiation source, but screened by a filter (Noviol "C"). A quartz-mercury arc (tube dimensions: 56 × 0.8 cm., power supply: a 15,000 volt, 30 milliampere transformer) was used as the ultraviolet light source.

Experiments

Inactivation of Fertilizin by X-radiation

In a typical experiment, a sample of egg water obtained as the supernatant from 50 cc. of eggs was divided into five equal parts. One part was kept as control and the other four samples were irradiated as indicated in Table I. After irradiation the agglutinating titer of each sample

Table I

Destruction of agglutinating power of Arbacia egg water by X-radiation

r units	Fertilizin titer	Per cent inactivation
0	2,048	—
2,800	1,024	50
5,600	256	87.5
16,800	2 (?)	99.9
28,000	0	100

was determined by serially diluting the samples in ½ dilutions with sea water and testing each dilution with an equal amount of 0.5 per cent sperm suspension. Titers are given as the greatest dilution at which agglutination is still microscopically perceptible. The units are, there-

fore, roughly 20 to 40 times greater than those employed by Tyler and Fox (1940).

As shown in the table, inactivation of this sample of fertilizin (titer 2,048) is practically complete with irradiation above 16,800*r*. Two other experiments gave similar results.

Richards and Woodward gave no information concerning the output of their X-ray apparatus. Since they used a very weak fertilizin solution and noted only a slight fall in agglutinin titer after 7.5 minutes of irradiation, it may be concluded that they used relatively low X-ray doses. Evans, Beams and Smith (1941) irradiated samples of *Arbacia* egg jelly (egg suspensions?) with 61,000*r* and 122,000*r*. The former sample gave light agglutination and the latter gave none. These values are in agreement with those presented here, assuming that dilute test sperm suspensions were used, since the same workers have shown, in *Arbacia,* that X-ray doses of 15,000*r* completely remove the jelly, which is identical with or contains the fertilizin (Tyler, 1940, 1941). Thus, an irradiated egg suspension is equivalent to an irradiated fertilizin solution of high titer.

Production of Univalent Fertilizin by X-Rays

As is well known (Lillie, 1919; Tyler, 1940), the agglutination of sea urchin sperm by egg water is a spontaneously reversible phenomenon. Furthermore, sperm which have been agglutinated by a sufficiently strong egg water will not, after reversal, reagglutinate upon further addition of fertilizin. Theoretically, sperm treated with fertilizin that had been converted to the univalent form should be equivalent to sperm that had reversed and so no agglutination should occur upon subsequent addition of normal fertilizin.

The sperm agglutinin titer of any fertilizin sample may be determined by serially diluting the sample and testing for agglutination with constant amounts of a sperm suspension, as previously outlined. If, after reversal of this agglutination, equal amounts of a test (normal) egg water are added to each dilution and the greatest dilution at which no agglutination occurs is recorded, a quantitative measure of the agglutination inhibiting power of the sample is obtained, which may be termed the inhibition titer of the sample. When both the agglutinin and inhibition titers of an irradiated sample and its unirradiated control are determined, using the same sperm suspension and test egg water solution, the formation of any univalent fertilizin through the treatment may be detected by a comparison of these values. It is thus not necessary to inactivate the fertilizin completely to test for the univalent form. This is desirable, in

that excessive irradiation might result in complete destruction of the fertilizin.

A strong fertilizin solution was prepared by extracting eggs in slightly acid sea water (pH 4.5). By this treatment 105 cc. of egg suspension gave 65 cc. of egg water and 40 cc. of jellyless eggs. After readjusting the solution to pH 8, a 5 cc. sample was removed and irradiated for five minutes at 5,600*r*/minute. Two drops of the irradiated solution and two drops of the control were diluted serially in tenfold steps with sea water, two drops of a 1 per cent sperm suspension were added to each dilution, and the dishes were allowed to stand until the agglutination in the undiluted control had reversed. Two drops of 1/10 dilution of the original control fertilizin were added and the dishes examined for agglutination.

TABLE II

Ability of X-rayed Arbacia fertilizin to react with sperm and thus inhibit subsequent agglutination of this sperm by test fertilizin

	Agglutinin titer	Agglutination after reversal of control upon addition of a test egg water to undiluted samples	Inhibition titer (Reciprocal of dilution of egg water at which test egg water first gave agglutination)
Control egg water.....	100,000	Negative	1,000
Irradiated egg water...	100	Negative	1,000
1,000 fold dilution of control egg water....	100	Positive	0

The irradiated egg water was found to have about 1/1,000 the agglutinin titer of the control. However, it was found to be just as effective as the control solution in preventing subsequent agglutination. The quantitative data are given in Table II.

It is of interest that no precipitate which might be attributed to this radiation was seen to form in this or any of the other X-rayed fertilizin solutions, even after prolonged exposure.

Inactivation of Strongylocentrotus Fertilizin by Ultraviolet Light

A stock solution of *Strongylocentrotus purpuratus* fertilizin was made up by extracting, with acid (pH 3) sea water a concentrated suspension of shed eggs, centrifuging and adjusting the supernatant to pH 8. Two 4 cc. samples were removed from this stock solution. One was irradiated and the other used as a control as previously described.

At various intervals of time equal portions of solution (four drops) were removed from the irradiated and control samples for titration of

sperm agglutinin activity. Titrations were made by adding equal amounts (two drops) of 1 per cent sperm suspension to equal amounts of the fertilizin diluted serially in ½ dilution steps. The titer, as in the case of *Arbacia,* was taken as the greatest dilution at which agglutination could be observed in two to four seconds under the microscope: Since the same 1 per cent sperm suspension, pipettes, etc., were used throughout, the titrations, as presented in Table III, are directly comparable. The results are typical of several experiments.

TABLE III

Destruction of agglutinating power of S. purpuratus fertilizin by ultraviolet light

Duration of irradiation	Agglutinin titer	Per cent inactivation
0	16,384[1]	—
60 min.	1,024	93.8%
90 "	256	98.4%
124 "	16	99.9%
154 "	0	100.0%
349 "	0	100.0%

[1] Average of the five controls, none of which differed more than one dilution step from this value.

As the data indicate, complete inactivation of the sperm-agglutinating property of this fertilizin solution was attained in 2.5 hours by the ultraviolet light source. Since both the controls and irradiated samples were allowed free access to air and were the same distance from the radiation source, evaporation and effect of ozone are ruled out as causal agents. Thermometers placed in the position of the control and irradiated samples indicated at most a 2° C. rise above room temperature, but never a difference of more than 1° C. in the two positions, so the inactivation found is attributed to the radiation from the arc.

Production of Univalent Fertilizin by Ultraviolet Light

All irradiated and control fertilizin samples recorded in Table III were tested for univalent fertilizin by adding one drop of a test fertilizin solution (stock solution diluted to 20 per cent) to each dish after reversal had occurred in the undiluted controls. As before, the inhibition titer is that dilution of the original egg water (irradiated or control) at which agglutination was first observed upon addition of the test fertilizin. Table IV gives the data for the sample irradiated for 349 minutes. This result is similar to that obtained with the other four samples which received lower radiation doses.

The data show less inhibition (one dilution step) for the irradiated than for the control fertilizin. The difference is within the error of the method (it was found in the other four determinations as well), and may be attributed to inaccuracies in making up the dilutions, to partial inactivation of the specific combining groups of the fertilizin by the ultraviolet treatment, or more probably to incomplete reversal of the initial

TABLE IV

Ability of ultraviolet light treated S. purpuratus fertilizin to react with sperm and thus inhibit subsequent aggutination of this sperm by test fertilizin

	Agglutinin titer	Agglutination after reversal of control upon addition of test fertilizin to undiluted samples	Inhibition titer (Reciprocal of dilution of egg water at which test egg water first gave agglutination)
Control for sample irradiated 349 min.....	16,384	Negative	8
Egg water irradiated 349 min.....	0	Negative	4

agglutination in the control. Microscopically "complete reversal" is an ideal condition seldom attained with strong egg water. Any small unreversed clumps of sperm obscure a faint reaction by the test egg water.

Experiments on the fertilizin of the sea urchin *Lytechinus anamesis* showed that it also lost its sperm agglutinating power and became univalent when treated with ultraviolet light.

DISCUSSION

An attempt has been made to give standardized data on the X-ray inactivation of *Arbacia* fertilizin and the ultraviolet light inactivation of *S. purpuratus* fertilizin. The limitations involved in the use of "dry" sperm as a standard for sperm concentration are fully appreciated. The concentration of spermatozoa in dry sperm varies from animal to animal and with the method used in obtaining the sperm. The standard is, however, a convenient one, and has been recognized by wide application. It is accurate enough to justify its use here.

Tyler (1941) has suggested that the well known reversal of sperm agglutination in sea urchins results from a splitting of the fertilizin molecule, leaving univalent fragments attached to the sperm; and that non-agglutinating fertilizin obtained through heating or enzymatic digestion likewise involves a splitting of the molecule to form univalent

fragments. The initial rise in agglutinin titer recorded by Richards and Woodward (1915) for X-rayed fertilizin, and by Tyler (1941) for heat or enzyme treated fertilizin, are interpreted as the initial step—a splitting of the highly multivalent molecule into two or more smaller multivalent fragments—in the production of univalent fertilizin. This initial rise in agglutinin titer was not observed in the present work, although it might have been found with lower dosages.

It is shown here that fertilizin treated with X-rays or ultraviolet light loses its sperm-agglutinating power, but retains all or nearly all of its ability to combine with sperm. This indicates that a destruction of all but one sperm-combining group per molecule of fertilizin is probably not the mechanism of this inactivation. On the contrary, the data favor rather strongly Tyler's view that the original multivalent molecule is split into fragments containing but one combining group per molecular fragment.

Heat, ultraviolet light, and X-rays are denaturating agents (Clarke, 1936; Arnow, 1936), and since fertilizin is probably a protein (Tyler and Fox, 1940) it is possible that univalent fertilizin is formed as a result of incomplete denaturation of this substance. On the basis of Tyler's explanation of univalence, this would imply that in denaturation there is initially a splitting of the molecule. Such an effect has been recorded by Svedberg and Brohudt (1938) for *Helix* haemocyanin. When solutions of this material were irradiated with ultraviolet light at pH 7.4 a splitting into half and into smaller molecules occurred, followed, with prolonged irradiation, by complete coagulation. Bawden and Pirie (1937) showed that heat denaturation of the tobacco mosaic virus protein resulted in a splitting off of nucleic acid. However, a number of simpler proteins undergo little if any change in molecular weight as a result of denaturation. Sanigar, Krejci and Kraemer (1939) report no change in the absorption spectrum or sedimentation behavior of purified serum albumen after X-radiation, although treatment with ultraviolet light resulted in the appearance of substances of low molecular weight. No cleavage of the egg albumen molecule was found by Fricke (1938) through the action of X-rays, although heating caused a slight hydrolysis, the effect being greater after irradiation. Likewise, Mirsky (1938) obtained no decrease in molecular weight in the heat or acid denaturation of trypsin. Obviously, no general rule may be formulated from the work on denaturation which may be applied to the inactivation of the agglutinating power of fertilizin and the simultaneous appearance of the univalent material but Tyler's view of a fragmentation of the fertilizin molecule is supported by some of the above work and is favored here as the simplest explanation for the results obtained.

Summary

1. The X-ray inactivation of *Arbacia punctulata* fertilizin reported by Richards and Woodward (1915) and by Evans, Beams and Smith (1941) is confirmed. Further data on this inactivation are presented in terms of Roentgen units, fertilizin concentration and sperm concentration.

2. It is shown that ultraviolet light is effective in the inactivation of *Strongylocentrotus purpuratus* and *Lytechinus anamesis* fertilizin.

3. *Arbacia* fertilizin which had been almost completely inactivated by X-rays, as judged by its ability to agglutinate sperm, lost none of its power to react with sperm. Thus, sperm treated with this fertilizin showed the same resistance to subsequent agglutination by untreated fertilizin as did sperm which had reversed after treatment with unirradiated (control) fertilizin.

4. *Strongylocentrotus* and *Lytechinus* fertilizins whose sperm agglutinating powers had been completely destroyed by ultraviolet light likewise retained their original sperm combining powers, as indicated by their ability to render sperm non-reactive to untreated fertilizin.

5. Tyler's (1941) view that fertilizin may be converted to a non-agglutinating, "univalent" form by splitting the fertilizin molecule into fragments, each having but one reacting group, is considered the most reasonable explanation for the results.

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